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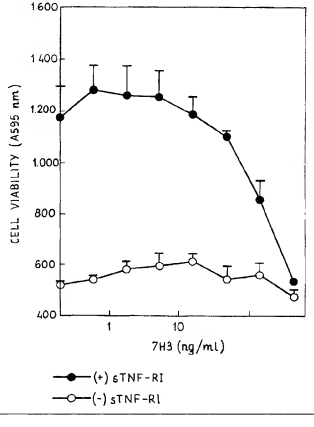
(57) Abstract

Monoclonal antibodies, and derivatives thereof, against TNF-RI receptors are described, having no cytotoxic effects on target cells, on the contrary providing an antagonist activity and the ability to neutralise the TNF cytotoxicity, to be used in diagnostic and clinical procedures as well as in TNF purification.



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MONOCLONAL ANTIBODIES AGAINST THE α-TNF TYPE I RECEPTOR, PROCESS FOR THE PRODUCTION AND USE THEREOF

The invention relates to a class of monoclonal antibodies against an epitope of the α-TNF (α Tumour Necrosis Factor) type I receptor, defined as TNF-RI, that is present either in a membrane associated, insoluble form or in a soluble form; said antibodies show an antagonist activity. The epitope is located either in coincidence or in proximity of the receptor TNF binding site; therefore, antibodies against the same have affinity characteristics, suitable to be used in pharmacology as TNF antagonists, as well as for measurements of TNF-RI at low concentrations in analysis systems and finally, as TNF-RI purifying system.

The TNF is a cytokine, that is mainly secreted by activated macrophages and was initially detected in serum of BCG (Bacillus Calmette Guérin) infected mice, because of its ability of inducing an haemorrhagic necrosis of some tumours in experimental animal models.

More recently, TNF was identified as one of the main inflammation mediators. Under special circumstances of acute and/or chronic production, TNF may cause pathologic conditions, such as septic shock, cerebral malarial fever, lipid dysmetabolism, known as cachexia, the last being typical in patients affected by some chronic and neoplastic infectious diseases. Further, it was found that TNF, together with other cytokines and lymphokines, is effective in immunoregulation, angiogenesis, cell differentiation and growth processes (Beutler, B. and Cerami, A., Ann.Rev.Immunol., 7, 625-655, 1989).

Many, if not all, of TNF induced effects are believed to be mediated by receptors on the membrane of

target cells. Two different receptors, named TNF-RI and TNF-RII, were recently purified and characterised (Smith, C.A. et al., Science, 248, 1019-1023, 1990; Schall, T.J. et al., Cell, 61, 361-370, 1990). The TNF cytotoxic activity is mainly mediated by TNF-RI. Moreover, soluble forms, corresponding to extracellular portion of both receptors and shoving a strong inhibiting action against TNF, were purified either from urine of healthy subjects or, in a larger extent, from urine of feverish or affected by chronic renal failure patients, or from serum of neoplasia affected patients (Nophar, Y. et al. Embo J., 9, 3269-3278).

In some pathologic states, such as septic shock (Waage et al., Lancet, i, 355, 1987), rejection of transplanted kidneys (Maury et al., J. Exp. Med., 166, 1132, 1987), parasitic infections (Scuderi et al., Lancet, ii, 1364, 1986) and various neoplasias (Balkwill et al., Lancet, ii, 1229, 1987), plasmatic levels of TNF increase considerably.

Consequently an obvious need arises to develop compounds able to neutralise the toxic effects induced by endogenous TNF, when the same is secreted in excess, such as in septic shocks or cerebral malarial fevers, by neutralising the TNF-receptor binding. An inhibition of this interaction could be obtained by using compounds able to bind to the receptor in competition with TNF. In particular, molecules binding to type I receptor, but not to type II receptor theoretically may be able to act as antagonist of activities mediated by type I receptor, as cytotoxicity; however they should not be able to act as antagonist of activities mediated by type II receptor, thus showing pharmacological selective properties.

In this context, the term "monoclonal antibodies against TNF-RI, that are TNF antagonist" means monoclonal antibodies, or derivatives thereof,

comprising recombinant antibodies, antibody fragments, that are able to identify and bind with high affinity to epitopes of TNF-RI, said epitopes are sterically close or are coincident with TNF binding sites, so that to act as effective antagonists of the TNF-receptor binding.

It is known that antibodies against receptors, driven to epitopes coincident with the hormone/receptor interaction site, could mime some of properties of the natural ligand, thus acting as agonist, while antibodies against different epitopes could merely prevent binding of ligand to the receptor, thus acting as antagonist.

The production of murine monoclonal antibodies against TNF-RI was reported in the literature. However, described antibodies are characterised in having an agonist activity, or a very poor antagonist activity. For example, monoclonal antibodies against TNF-RI and able to induce cytotoxic effects even when TNF is absent (agonist), were described by Engelman et al., J. Biol. Chem., 265, 14497, (1990). A monoclonal antibody, that is able to inhibit TNF cytotoxic and cytostatic activities (antagonist) was described on the other hand by Thoma, B. et al., J. Exp. Med., 172, 1019 (1990). However this antibody (H398) is able to act as antagonist of TNF (1 ng/ml) cytotoxic activity on U937 cells only at very high concentrations, i.e., ranging from 10 to 100 μg/ml (see fig. 4, p. 1021 of the above mentioned publication). Furthermore, said H398 antibody is able to act as antagonist of TNF cytotoxic activity at high concentrations, i.e in the range of 10 μg/ml (see table 1, page 1022, cited publication), only when the TNF concentration ranges from 1 to 10 ng/ml, thus showing a poor antagonist activity. Similarly, Espevik et al., J. Exp. Med., 171, 415, (1990) described an antibody (htr-5) that is able to act as a weak

antagonist action against the TNF cytotoxicity on U937 cells. Particularly, as shown in fig. 5 at page 421, the htr-5 antibody, at a concentration of 10 μg/ml, acts only as a partial antagonist of TNF, ranging from 1 to 10 ng/ml, cytotoxicity, since more than 50% of the cells are killed, even in the presence of a large excess of antibody.

The skilled in the art could easily understand that the effective and useful ability to act as antagonist of TNF cytotoxicity may depend upon different factors; among them: a) the antibody binding affinity to the cell receptor (defined by the affinity constant); b) the binding and dissociating rates (defined by the association and dissociation constants); and c) the ability to interfere with the signal transduction, e.g., by impairing the TNF-induced clustering of receptor molecules, that is essential to transmit into the cell the toxicity signal.

In order to develop antagonist drugs, an obvious need arises to obtain monoclonal antibodies, having an effective capacity to inhibit at low doses the TNF cytotoxic action.

The author isolated and used a novel monoclonal antibody, which is able to recognise and bind the TNF-RI receptor and to be an effective TNF cytotoxic action antagonist at low doses, specially when assayed on human cells. Further, this antibody is able to bind the TNF-RI receptor with a fast kinetics and require no previous incubation, as opposite to antibodies of the prior art, to act as antagonist of TNF cytotoxicity. Further, this antibody is able to bind the TNF-RI receptor with kinetics similar to liquid state kinetics, even when it is fixed on a solid matrix and/or denatured by detergents. This allows to use advantageously the antibody for TNF-RI receptor isolating or detecting methods, wherein affinity

chromatography or western blotting procedures are used. Finally, the antibody surprisingly is able to identify and bind unknown high molecular weight forms of TNF-RI receptor, which were identified in the urine.

The antibody of this invention is obtained through cell fusion and somatic hybridisation between mouse no-secreting myeloma cells (i.e., unable cells to secrete immunoglobulin light or heavy chains) and spleen cells from mice previously immunised with soluble TNF-I receptor (sTNF-RI). BALB/c mice are immunised, using high purified sTNF-RI preparations, and splenocytes are fused with myeloma no-secreting cells, unable to survive in culture media containing aminopterin. Examples of myeloma cells are: X63-Ag8653, NS-O, SP2/O-Ag14, NS-1 cells. Such cells are generally available, being supplied by public organisations, such as ECACC (European Collection of Animal Cell Culture, Porton Down, Salisbury, SP4 0JG, U.K.). The cell fusion is carried out in presence of polyethylene glycol.

The TNF-RI preparation either from human urine or from supernatants of cells transfected by the coding gene the extracellular domain of the membrane receptor, is well known in the art. Both natural and recombinant preparations could be used as antigens. Preferably, the immunising process uses low doses of highly purified natural TNF-RI to be administered to BALB/c mice. An immunisation and cell fusion process, that is suitable to generate high affinity antibodies against TNF-RI in BALB/c mice, comprises the following administration cycle at two week intervals:

a) 5 μg of sTNF-RI in Freund's Complete Adjuvant, by means of four subpelmatic injections (half dose), followed by a number of subcutaneous injections on different locations (the other half dose);

b) a similar dose of TNF-RI in Freund's Incomplete Adjuvant to be subcutaneously administered only;

c) a similar of TNF-RI in PBS buffer to be intraperitoneally (i.p.) administered; and finally the same PBS buffered dose i.p.

Spleens of animals showing high antibody rates (higher than 1:100.000 in serum), are picked up three days after the last administration. Then splenocytes are fused with NS-0 myeloma cells at a 1:5 ratio. After the fusion, cells are cultivated in HAT medium containing hypoxanthine, aminopterin and thymidine for 8-15 days, and supernatants tested for the presence of antibodies against sTNF-RI, by means of an ELISA assay, allowing to select high affinity antibodies. Such assay consists of:

a) capturing of murine antibodies by coated wells with goat anti-mouse IgG immunoglobulins;

b) incubating with biotinilated sTNF-RI; and

c) detecting the reaction with streptavidin peroxidase and chromogen substrates.

Then resulting hybridomas are cultivated according to conventional methods and antibodies purified by methods known to those skilled in the art and are, accordingly, out of the scope of this invention per se. The author, among the obtained antibodies, selected an antibody having the highest antagonist level by means of a cytotoxicity assay, wherein TNF is incubated with U937 cells in presence and in absence of different monoclonal antibody doses. Therefore it is possible to identify and select antibodies that, other than being anti-sTNF-RI, also show an action against the membrane form of TNF-RI.

Accordingly, it is an object of the invention an anti-TNF-RI receptor monoclonal antibody, or a derivative thereof, having no cytotoxic effects but

antagonist effects, able to neutralise the TNF cytotoxicity, even when used at low doses.

In the context of this invention, the term "derivative of monoclonal antibodies against TNF-RI" mean molecular forms, comprising one or more antibody complementary determining regions (CDRs), chemically or genetically manipulated for various purposes, such as, e.g., to reduce the immunogenicity or to improve pharmaceutical properties thereof. Examples of derivatives of the antibody against TNF-RI are:

a) antibody molecules, wherein either CDRs or whole antibody variable regions were transferred into antibody molecules of a different isotype or into human molecules; and

b) antibody molecules consisting of antibody variable regions, genetically manipulated in order to obtain single or double chain fragments that could be expressed in prokaryotic systems.

In a preferred embodiment of this invention, the monoclonal antibody against TNF-RI is an IgG1, more preferably is the antibody, known as 7H3, which was deposited at DSM under the accession No. ACC2123.

It is a further object of the invention an immunology assay method to identify qualitatively or quantitatively TNF-RI from fluids or biologic tissues, comprising, as specific ligand, the antibody according to the invention and revealing means to detect the reaction between said TNF-RI and said specific ligand.

Preferably, said immunology assay method is carried out either in a liquid or in a solid phase, more preferably is either a direct or a competitive method, and most preferably said revealing means comprise either enzyme, radioactive, fluorescent or chemioluminiscent tracers, or colloidal metals, or some other detecting system known in the art.

In a preferred embodiment of this invention, said method comprises the use of the 7H3 antibody.

According to this invention, said method is able to distinguish the soluble, membrane-associated, or free TNF-RI from TNF-RI which is not associated to TNF.

It is a further object of this invention a method to purify TNF-RI from biologic fluids through affinity chromatography on support immobilised 7H3 antibodies. Preferably, said support is comprised in the group of agarose, glass beads, cellulose, polyacrilamide, etc. Methods to immobilise antibodies to said supports are known to those skilled in the art.

This invention will now be described according to some preferred embodiments with reference to the alleged figures, wherein:

fig.1 shows an ELISA assay diagram, using a polyclonal serum against sTNF-RI;

fig.2 shows a diagram of the 7H3 antibody binding at different concentrations of biotinilated sTNF-RI, wherein: B/BamX is the ratio between different absorbances of different concentrations of biotinilated sTNF-RI and the absorbance of the biotinilated sTNF-RI concentration of 400 ng/ml;

fig.3 is a cell viability test;

fig.4 is a cell viability test;

fig.5 is a cell viability test;

fig.6 shows the sTNF-RI elution curves from a TNF-agarose column (A) and from a 7H3-agarose column (B); and

fig.7 is a schematic view of an electrophoresis gel of sTNF-RI purified from urine (A) and after a western blot with the 7H3 antibody (B).

EXAMPLE 1 - Immunisation and production of monoclonal antibodies against sTNF-RI

BALB/c (Charles River) female mice are immunised by subcutaneous injection of 2.5 μg/ml of 1:1 emulsified TNF-RI in CFA (Freund's Complete Adjuvant), as well by subpelmatic injections of the same composition. After two weeks 5 μg/ml of TNF-RI in CFA are administered subcutaneously. Two booster doses by peritoneal injections of 5 μg/ml of TNF-RI in 200 μl of PBS are administered at two week intervals. After three days from the last administration of the booster dose, mice are killed, spleen cells isolated and fused with NSO myeloma cells, using standard procedures (Galfré, G. and Milstein, C., Methods Enzymol., 73, 1-46, Academic Press, NY, 1981).

A screening for the presence of anti-TNF-RI antibodies in the hybridoma culture media through an ELISA assay is carried out as follows: PVC microtitration plates are filled with a solution of GAM (IgG) in PBS (50 μl per well), overnight at 4°C, and fixed with 200 μl of PBS at 1% w/v of BSA, for 2 hours at 37°C. After washing with PBS-T, each well is filled with 50 μl of 1:1 PBS-BT diluted samples and incubated for 90 minutes at 37°C. Then each well is added with 50 μl of 1:10.000 biotinilated TNF-RI in PBS-BT and incubated for 1 hour at 37°C. After a further washing with PBS-BT, plates containing 50 μl/well of 1:2000 STV-HRP in PBS-BT, are incubated for 1 hour at 37°C. Then, a further washing is carried out, followed by an incubation with 100 μl of a chromogenic ABTS solution for 30 minutes at 37°C. The 405 nm absorbance of each well is measured, using a model 2550, EIA reading instrument (Biorad, Richmond, California 94804).

The isotypes are defined using a "Mouse Typer Iso-typing Kit", according to instruction of the Manufacturer (Biorad, Richmond, California 94804). The 7H3 monoclonal antibody (IgG1) and polyclonal anti-TNF-RI IgGs are respectively purified from hybridoma

culture medium and from immunised sera, through affinity chromatography procedure on protein A agarose (Schmidt, C., J. Biotechnol., 11, 235-242, 1989). The final products has a purity level higher than 90%, as indicated by electrophoresis on polyacrilamide gel in SDS (SDS-PAGE).

As shown in fig.1, TNF-RI is able to induce a strong immune response, as higher titres than 1:100.000 are obtained. A clone is selected from the fusion of preimmunised mouse spleen cells with NSO myeloma cells, named 7H3, and deposited at DSM under the accession No. ACC2123, which produces an antibody against TNF-RI. Isotyping tests indicated that 7H3 is a IgG1 kappa.

The binding affinity of different biotinilated TNF-RI concentrations with antibodies in solid phase is measured. The sTNF-RI is biotinilated as follows: 20 μl of 0.5 mg/ml of TNF-RI in water buffered with 2.2 μl of sodium carbonate, pH 8.8, and with 2.2 μl of D-biotinil-6-aminohexanoic acid N-hydroxysuccinimide ester (5mg/ml in DMSO). After incubating for four hours at room temperature, the solution is added with 2.5 μl of 1 M lysine and incubated for four hours. The product is maintained at -20°C.

As shown in fig. 2, the 7H3 antibody provides a high binding affinity, as 50% of binding sites are saturated by appr. 0.1 nM of biotinilated TNF-RI.

The topographic relationship between TNF-RI sites recognised by the 7H3 antibody and by TNF is investigated through cross-competitivity tests. The biotinilated sTNF-RI binding to 7H3 antibodies in solid phase in presence and absence of TNF is measured. The results of table 1 show that TNF is able to inhibit the 7H3 antibody binding to sTNF-RI.

Table 1

Biotinilated sTNF-RI binding to 7H3 antibodies in solid phase in presence and absence of 20 ng/ml of TNF

Antibody	Absorbance at 405 nm	
	- TNF	+ TNF
7H3	1.439	0.527

EXAMPLE 2 - Cytotoxicity assays

Human U937 and murine LM cell lines (ATCC, Bethesda, Maryland, US), that are sensitive to human TNF cytotoxic action, are used to evidentiate 7H3 antibody agonist and antagonist actions.

The TNF cytotoxic action on LM murine cells is tested through a cytolysis method, based upon the vital cell stain by MTT, as described by Coffman and al., "Lymphokine Res.", 7, 371, 1988.

The TNF cytotoxic action on U937 human cells is tested as follows: U937 cells are cultivated for one day on 96 well plates (200.000 cells/well in 50 μl) in RPMI 1640 culture medium, containing 10% of FCS and 1% of glutamine. Then 50 μl of TNF solution at various concentrations (from 50 to 3.15 μg/ml) are added to the culture medium in each well, and a further incubation is carried out for two days. Then 50 μl of a MTT solution at 5 mg/ml are added, followed by an incubation for 4 hours at 37°C in 5% CO_2 environment; DMSO is added (200 μl in each well) to dissolve the dyed product, and the 595 nm absorbance in each well is measured (Biorad).

The agonist action is defined by measuring the cell viability after an incubation in presence of antibodies and in absence of TNF, while the antagonist action is defined by measuring the cell viability after having incubated in presence of both 7H3 antibody and TNF. Results in fig.3 show that the 7H3 antibody has no

cytotoxic effects on each cell line. On the contrary, the 7H3 antibody is effective in opposing the TNF cytotoxic action on U937 cells, but is not effective on LM cells, even with no preincubation with cells, therefore suggesting that its binding to the membrane receptor develops quickly (fig.4).

Also the 7H3 antibody ability to neutralise the sTNF-RI inhibition activity is measured. Accordingly, the LM cell viability after an incubation with mixtures of TNF, sTNF-RI and 7H3 at various concentrations is measured, as the 7H3 antibody does not interfere with the interaction between TNF and LM cell receptors. As shown in fig.5, the 7H3 antibody is able to effectively neutralise the sTNF-RI inhibiting activity.

These findings indicate that the 7H3 antibody binds a TNF either membrane or soluble form receptor epitope. The lack of any antagonist activity on murine LM cells suggests that the binding is species specific.

EXAMPLE 3 - ELISA assay using sTNF-RI

Polyclonal IgGs are biotinilated substantially as described for TNF-RI, except that the final product is dialysed against PBS overnight at 4°C. Products are further diluted to 3 ml with PBS and maintained at -20°C.

sTNF-RI in urine extracts and in culture media is detected as follows:

PVC microtitration plates are filled with a solution of the 7H3 antibody in PBS (100 µl/well) at 10 µg/ml and incubated overnight at 4°C. All the following steps are carried out at room temperature. After three washing with PBS-T, wells are blocked with PBS, containing 0.5% w/v of BSA and 0.05% of Tween 20 (stop buffer: 200 µl/well, 2 hours) and washed with PBS-T. Standard sTNF-RI, or sample solutions, diluted with PBS with 50% FCS, are mixed with biotinilated polyclonal IgGs against sTNF-RI at a 2:1 ratio (1:400 in stop

buffer) and a preincubation is carried out for 1 hour. 150 µl of each mixture are added to each well and a further incubation is carried out under gentle stirring for 2 hours. Then plates are extensively washed with PBS-T and incubated with a STV-HRP solution for one hour (Janssen, 1:4000 in stop buffer). Following a final washing with PBS-T, each well is incubated with 100 µl of a chromogenic ABTS solution for 1 hour, followed by the 405 nm absorbance reading.

EXAMPLE 4 - Purification of sTNF-RI from urine extracts

sTNF-RI from urine is purified using two methods: the first one (Method A) through an affinity chromatography on an agarose-TNF column; and the second method (Method B) through an affinity chromatography on an agarose-7H3 column. Both agarose-TNF and agarose-7H3 columns (1 cm x 3 cm) are prepared by coupling 2 mg of TNF or 10 mg of 7H3, respectively with CH-activated Sepharose® (Pharmacia), according to Suppliers' instructions; a coupling efficiency higher than 90% is obtained.

Method A. A crude urine extract (1,6 l), corresponding to 160 l appr., is dialysed overnight against PBS and concentrated to 5 ml through an ultrafiltration on a PTGC membrane (cut off 10.000 NMWL) on a Minitan (Millipore) equipment. The product is loaded on the agarose-TNF column (flow speed 40 ml/h) and washed out with PBS. The elution is carried out using a 0.2 M glycine buffer, pH 3.5. Peak containing fractions are collected, joined and further purified on a Pro-RPC HR5/10, reverse phase HPLC column (Pharmacia), under conditions as follows: buffer A: 0.1% of trifluoroacetic acid in water; buffer B: 0.1% of trifluoroacetic acid in acetonitrile; 0% of B for 20 minutes; linear gradient 0-70% for 50 minutes; 100% of B for 20 minutes, flow speed 0.3 ml/h. Fractions are collected and dried with a Speed-Vac (Savant)

concentrator. The product is diluted into 100 µl of distillated water and maintained at -20°C.

Method B. sTNF-RI from urine extracts is purified under the same conditions as for Method A, except the use of a agarose-7H3 column. sTNF-RI is eluted using 0.2 M glycine buffer, pH 2.8.

As shown in fig.6, eluted products from agarose-TNF and agarose-7H3 columns have different compositions: three major peaks appear in the agarose-TNF purified products (chromatography curve A), while only one peak appears in the agarose-7H3 purified product (chromatography curve B). Measurements of TNF neutralising activity of different fractions through a cytolysis assay on LM cells show that peaks 1 and 2 of curve A, as well as peak 1 of curve B, do contain TNF inhibitors; whereas peak 3 of curve A does not. Further, only peaks 1 of both curves contain sTNF-RI, as resulted by an ELISA assay.

The area of Peak 1 area is appr. 20% of the total area of curve A and more than 90% of the total area of curve B, suggesting that the 7H3-agarose column is more effective than the TNF-agarose column for purifying.

A SDS-PAGE analysis of peak 1 products show, in both cases, a major band (more than 90%) of appr. 31.000 dalton.

20 ng/ml appr. of both products are able to neutralise at 50% the toxicity of 250 pg./ml of TNF (with LM cells), thus indicating that a biologically active form of sTNF-RI is obtained.

EXAMPLE 5 - Characterisation of purified sTNF-RI on agarose-7H3 column

SDS-PAGE electrophoresis on 8-25 or 10-15 Phast-Gel gradients (Pharmacia) are carried out under reducing and not reducing conditions, according to Supplier's instructions.

Proteins are transferred onto a nitrocellulose membrane (Biorad), for 15 minutes at 15°C, in 25 mM Tris-HCl, 192 mM glycine, pH 8.3, 20% of methanol, as transfer buffer, at 25 mA. In order to detect sTNF-RI, membranes are washed using 100 mM Tris-HCl, 150 mM sodium chloride, pH 7.5 (TBS) and blocked with 0.5% of blocking reagent (Boehringer) for 1 hour at room temperature. After having been quickly washed with TBS, membranes are dipped into a solution of 2 μg/ml of 7H3 antibody in TBS-BR with 3% of preimmune goat serum (NGS) and 0.1% of BSA (TBS-BNRB) for 1 hour. Membranes are washed three times with TBS-BNRB, dipped in a STV-AP/TBS-BNRB (1:500) solution for 1 hour and washed again with 100 mM Tris-HCl, 100 mM NaCl and 50mM $MgCl_2$, pH 9.5. Alkaline phosphatase is detected by dipping the membrane into a chromogenic solution of BICP/NBT, as described by Harlow, E. and Lane, D. Antibodies: a Laboratory Manual, 407, CSH, New York, 1988.

As shown in fig.7, the 31 kD band reacts with the 7H3 antibody. Furthermore other bands, ranging from top of the gel up to a 60 kD molecular weight, are detected using this antibody, thus defining new epitopes of urinary TNF-RI precursors, or multimeric forms, antigenetically related to sTNF-RI and not yet described.

This invention was described with reference to some preferred embodiments, but not in a limitative way; it is understood that variations and/or modifications could be made by those skilled in the art without lost of scope of protection, when recognised as equivalent of what has been described here.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5; 7; 10; 17; , line 18; 22 ; 12; 13

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution: DSM - Dutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*:
Mascherocer Weg 1 B
D-3300 BRAUNSCHWEIG / GERMANY

Date of deposit	Accession Number
APRIL 15, 1993	DSM ACC2123

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

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CLAIMS

1. An anti-TNF-RI receptor monoclonal antibody, or a derivative thereof, characterised in having no cytotoxic effect and in having antagonist effects, able to neutralise the TNF cytotoxicity, even when used at low doses.

2. An anti-TNF-RI receptor monoclonal antibody, or a derivative thereof, according to claim 1, characterised in being an IgG1.

3. An anti-TNF-RI receptor monoclonal antibody, or a derivative thereof, according to claim 2, characterised in being the 7H3 antibody, deposited at DSM under the accession No. ACC2123.

4. An immunology assay method to identify qualitatively or quantitatively TNF-RI from fluids or biologic tissues, comprising, as specific ligand, the antibody according to any of previous claims; and revealing means to detect the reaction between said TNF-RI and said specific ligand.

5. An immunology assay method according to claim 4, characterised in being in a liquid or in a solid phase.

6. An immunology assay method according to claim 5, characterised in being either a direct or a competitive method.

7. An immunology assay method according to claim 6, characterised in that said revealing means comprise either enzyme, radioactive, fluorescent or chemioluminiscent tracers, or colloidal metals.

8. An immunology assay method according to claim 7, characterised in that said specific ligand is the 7H3 antibody.

9. An immunology assay method according to claim 8 characterised in being able to distinguish the

soluble, membrane-associated, or free TNF-RI from TNF-RI which is not associated to TNF.

10. A method to purify TNF-RI from biologic fluids through affinity chromatography on support immobilised 7H3 antibodies according to claim 3.

11. A method to purify TNF-RI according to the claim 10, wherein said support is comprised in the group consisting of agarose, glass beads, cellulose, polyacrilamide.

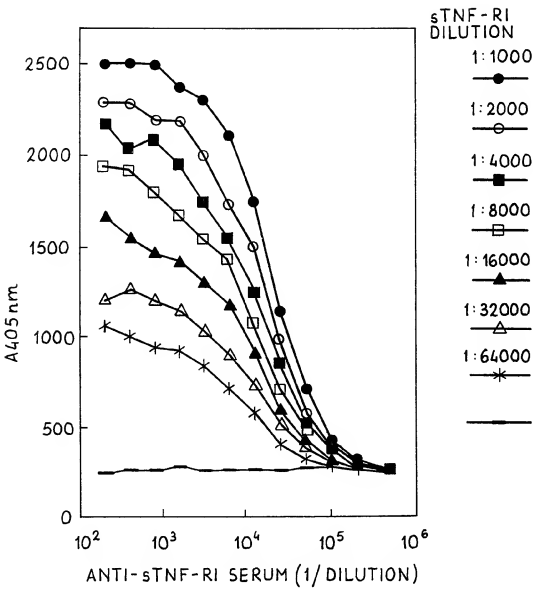
sTNF-RI
DILUTION
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
2500
2000
1500
1000
500
0
A405 nm
10^2
10^3
10^4
10^5
10^6
ANTI-sTNF-RI SERUM (1/DILUTION)

FIG. 1

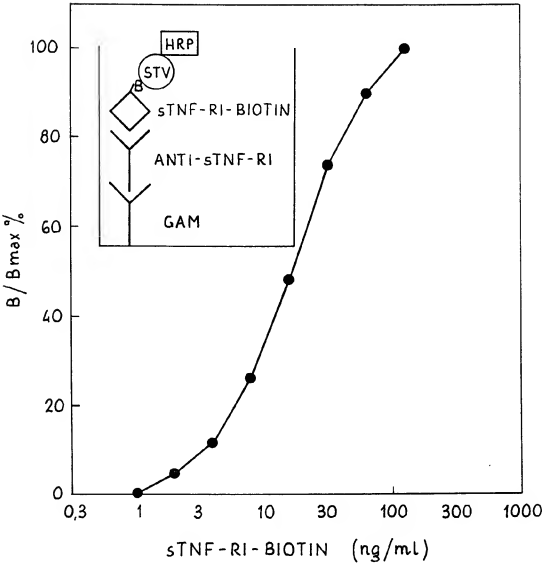
HRP
STV
B
sTNF-RI-BIOTIN
ANTI-sTNF-RI
GAM
B/Bmax %
100
80
60
40
20
0
0,3
1
3
10
30
100
300
1000
sTNF-RI-BIOTIN (ng/ml)

FIG. 2

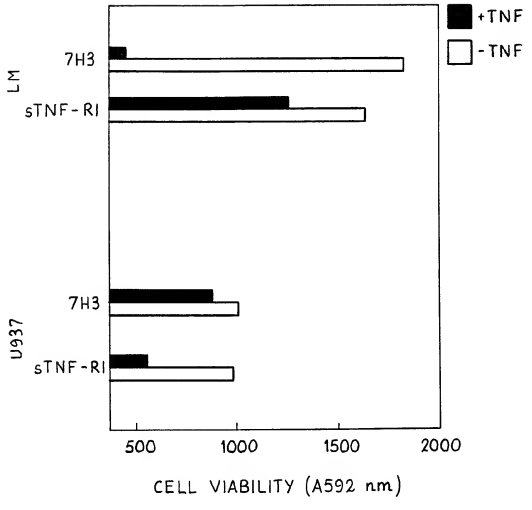
+TNF
-TNF
LM
7H3
sTNF-RI
U937
7H3
sTNF-RI
500
1000
1500
2000
CELL VIABILITY (A592 nm)

FIG. 3

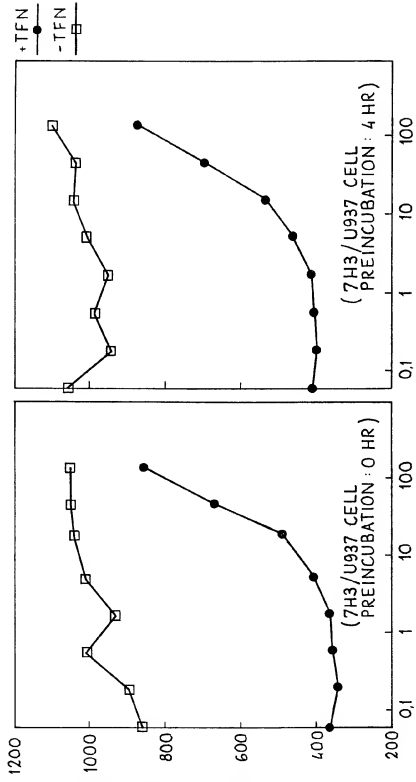
CELL VIABILITY (A595 nm)
1200
1000
800
600
400
200
0,1
1
10
100
(7H3/U937 CELL PREINCUBATION : 0 HR)
(7H3/U937 CELL PREINCUBATION : 4 HR)
+TFN
-TFN
7H3 (ng/ml)

FIG. 4

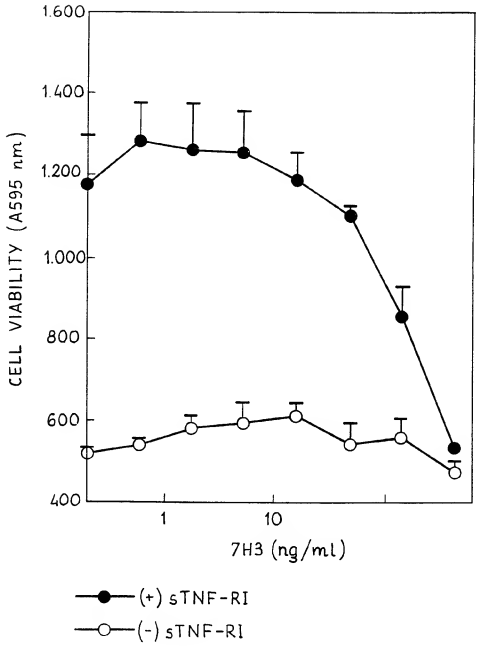
1.600
1.400
1.200
1.000
800
600
400
CELL VIABILITY (A595 nm)
1
10
7H3 (ng/ml)
(+) sTNF-RI
(-) sTNF-RI

FIG. 5

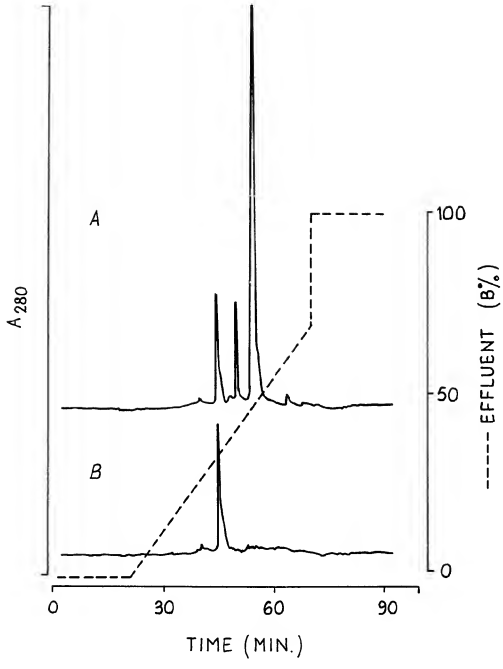

FIG. 6

FIG. 7